THE CHROMOSOMES OF SEA URCHINS, ESPECIALLY ARBACIA PUNCTULATA; A METHOD FOR STUDYING UNSECTIONED EGGS AT FIRST CLEAVAGE[1]

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Previously, there has been no truly adequate method for studying the chromosomes of sea urchin eggs, even when sectioning procedures were utilized. With classical methods, such as acetic orcein, carmine and the Feulgen techniques, the cytoplasm stains rather intensely, which tends to obscure the chromosomes. Moreover, the chromosomes are very small and numerous. Consequently, the chromosome number and morphology have never been determined with certainty in *Arbacia punctulata* and in many other marine invertebrates that have large, yolky eggs.

The present paper describes a simple and relatively rapid procedure that has been developed to examine the chromosomes and nuclear events of the early cleavage divisions of unsectioned eggs by a selective staining of the chromatin. With this method the chromosome number of *A. punctulata* has been established and that of the sand dollar, *Echinarachnius parma*, closely approximated. Also included is a description of the morphology of the chromosomes of the former species. A preliminary report has been published previously (Auclair, 1964).

The Method

Shedding of the eggs of both *A. punctulata* and *E. parma* was induced by injection of 0.5 ml. of 0.5 *M* KCl solution into the peritoneal cavity (Harvey, 1956). Dry sperm preparations were prepared by removing the testes and storing them at 4° C. in a dry stender dish.

For optimal results the fertilization membranes were removed. Exactly two minutes following insemination, mercaptoethylgluconamide was dissolved in the egg suspension to a final concentration of approximately 10^{-3} *M* (Harris, 1961). The loosened membranes were stripped off 15 minutes later by straining the eggs through a 25-mesh silk cloth. To prepare the eggs for metaphase chromosome spreads, colchicine was added about 20 minutes prior to the first cleavage division, followed by hypotonic treatment in 50% sea water 5 minutes before fixation.

The eggs were fixed in 50% ethanol at 4° C. for a minimum of one-half hour. Actually, any dehydrating agent, such as acetone, may be used. The specimens may be stored in the fixative for several weeks without any apparent impairment of staining quality.

For the staining procedure the fixative was decanted and replaced directly.

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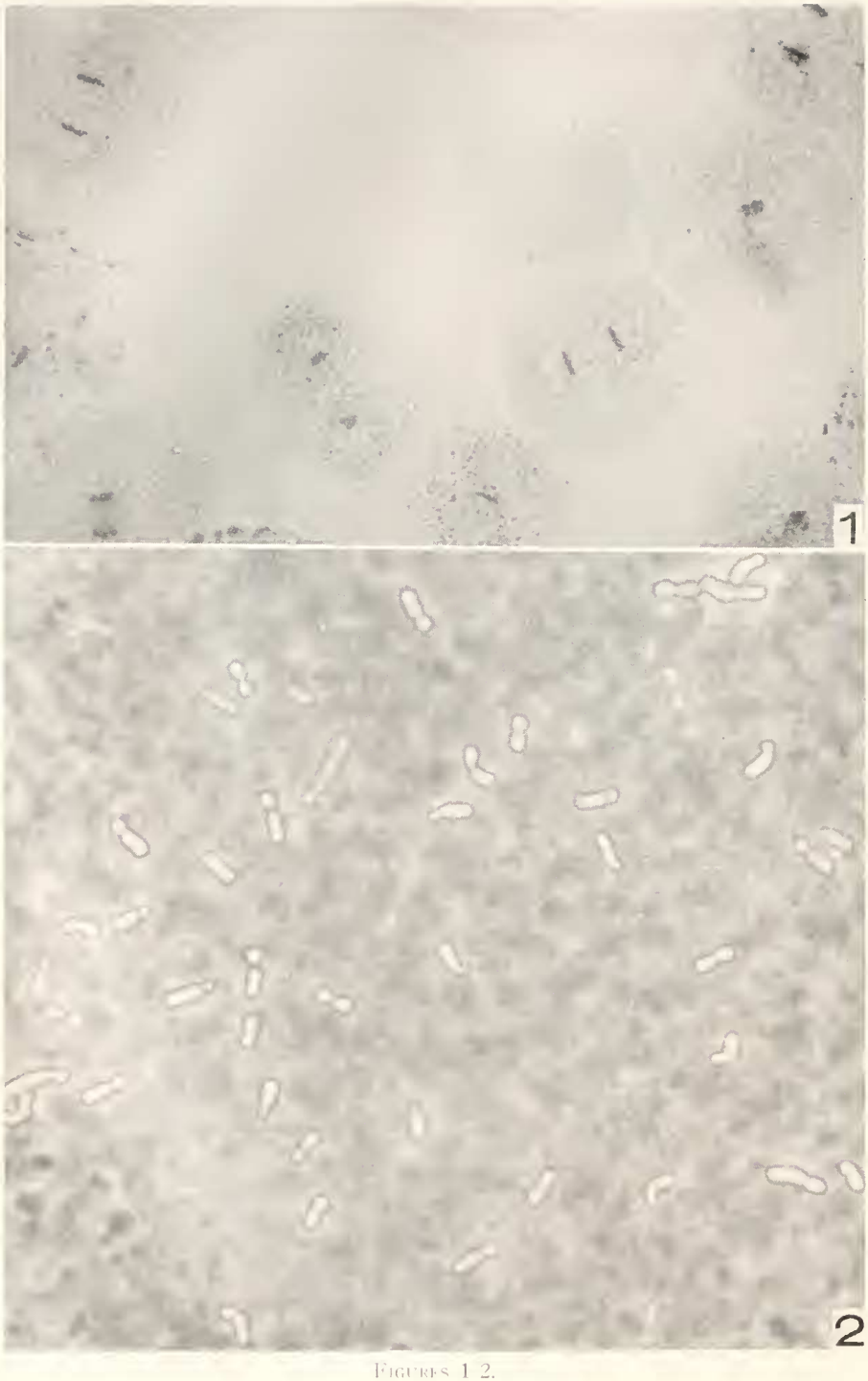


FIGURES 1 2.

without rinsing in distilled water, with a solution made by dissolving 0.1 gm. of adenosine 5′-monophosphate (AMP) in 20 ml. of 0.2 *M* Tris-maleate-NaOH buffer at a pH of 5.0 to 5.5. A number of different phosphates were used at an equivalent concentration, but the best results were obtained with organic phosphates, particularly AMP, that are not hydrolyzed to any extent by cellular phosphatases. To the buffer-organic phosphate mixture was slowly added 1 ml. of 1% $Pb(NO_3)_2$. The fine lead hydroxide precipitate that often appeared was removed by either filtration or centrifugation. The pH of the medium controls the speed of the reaction; the lower the pH the more rapid is the reaction, but background cytoplasmic staining is also increased at low pH. Incubation time has to be determined for each species. For *Arbacia* eggs, the optimal time for exposure to the medium was 1 to 1½ hours at 37° C., whereas for the sand dollar eggs, which are about twice as large as *Arbacia* eggs, several hours were required.

The eggs were then rinsed thoroughly with several changes of distilled water to remove excess lead ions, and then placed in 50% ammonium sulfide for several minutes. Following another rinsing the eggs were mounted in a water-soluble mounting medium or squashed in distilled water, making spread preparations of the chromosomes.

Nature of the Staining Reaction

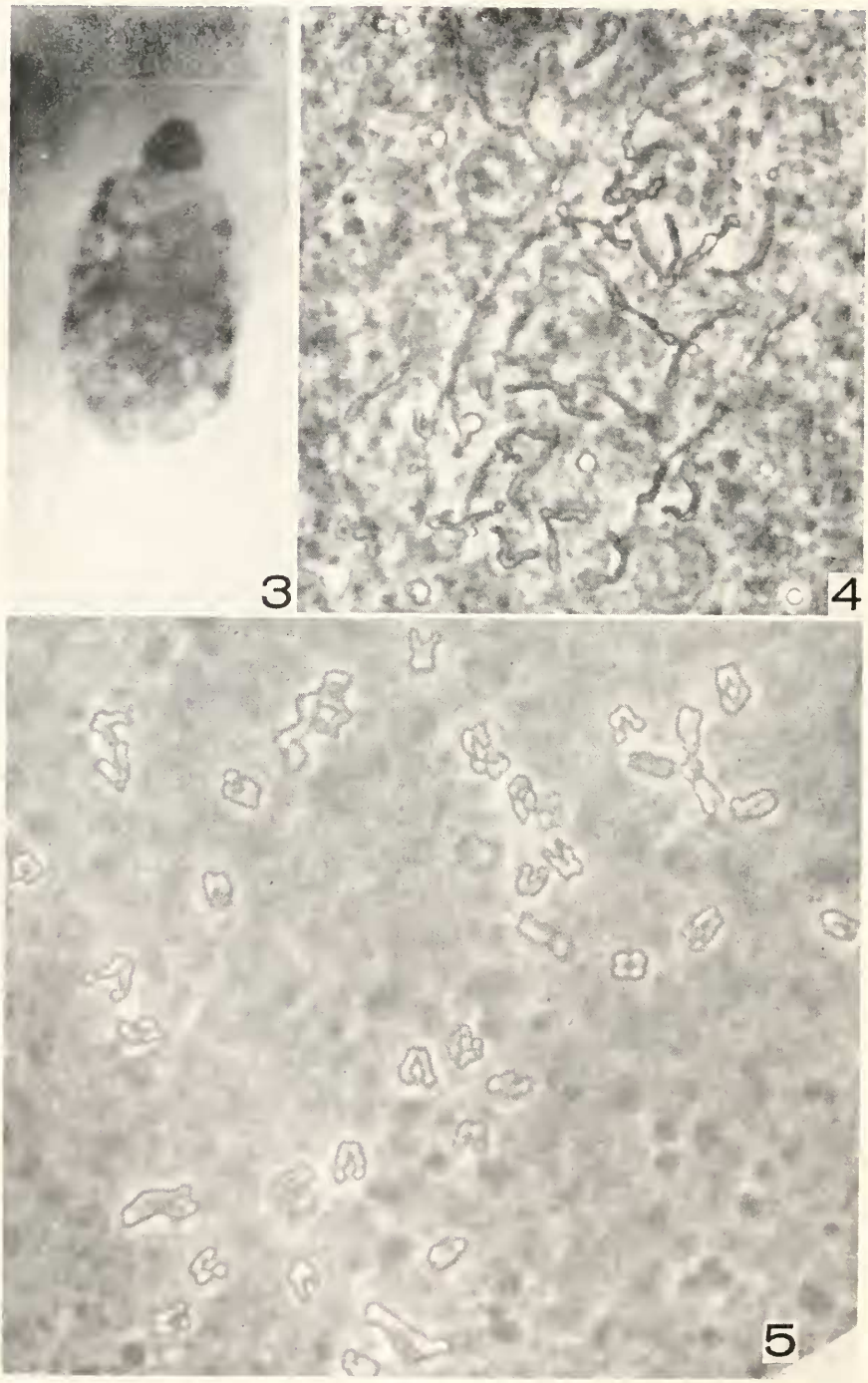
The staining reaction is a modification of the Gomori phosphatase technique, except that enzymatic activity is absent and only the nuclear structures are demonstrated. No chromosome staining occurred if either the lead or organic phosphate was absent. Any possibility of enzymatic hydrolysis was eliminated by the fact that when inhibitors of 5′-nucleotidase or acid phosphatase, 0.01 *M* $Ni(Cl)_2$ or 0.01 *M* NaF, respectively, were added to the lead-AMP solution, the staining intensity was not reduced. The reaction, moreover, is not specific for polymerized DNA. Digestion of the eggs in deoxyribonuclease (0.2 mg. per ml. of either 0.2 *M* Tris-maleate or phosphate buffer at pH 7.2 for four hours at 37° C.) resulted merely in a slight weakening of the staining intensity.

Further insight into the staining mechanism is provided by the following experiment. Ethanol-fixed eggs were placed for one hour (37° C.) in separate solutions of 0.2 *M* Tris-maleate buffer at pH 5.0 containing either 0.05% $Pb(NO_3)_2$ or 0.01 *M* AMP. Following a brief rinsing in distilled water, the lead-treated eggs were placed in buffer-AMP medium, and the AMP-exposed eggs were transferred to the buffer-lead solution. Only those eggs first exposed to the lead and then to the organic phosphate media gave a positive reaction. Inorganic phosphate used in place of AMP gave negative results in every case.

On the basis of these tests it seems probable that the lead ions diffuse into the partially dehydrated chromatin material and attach by ionic bondings to phosphate and other negatively charged sites. Organic phosphates, such as AMP, also diffuse inward and bind ionically with both lead and basic protein, thus anchoring the lead and reducing its susceptibility to removal by subsequent rinses. The sulfide

Figure 1. A general view of unsectioned *Arbacia* eggs in various stages of mitosis. Such preparations may then be squashed to make chromosome spreads. Magnification, approximately 400 ×.

Figure 2. Pre-metaphase chromosome spread of a colchicine-treated *Arbacia* egg in the first cleavage division. Oil immersion-phase contrast; magnification, approximately 2000 ×.



FIGURES 3–5.

ions then replace the phosphate groups from the lead ions, resulting in the precipitation and deposit of black lead sulfide at these sites. Thus, the term "staining" is not used in an exact sense, since no true dyeing process occurs.

Nuclear and Chromosomal Observations

By this method the sequence of nuclear events from fertilization through the first two cleavage divisions has been examined in unsectioned material. Background staining tends to be greater in the cytoplasm prior to the formation of the mitotic figure of the first cleavage division.

Syngamy and early prophase. The process of syngamy is revealed in striking fashion. The chromatin of the male pronucleus undergoes marked condensation into very densely staining structures as the two pronuclei come into contact (Fig. 3). Then the male pronucleus flattens itself against the contour of the female pronucleus and gradually the nuclei undergo fusion.

In early prophase the extended chromatid fibers may be seen coiling into elongated chromosomes (Fig. 4). The chromosomes then gradually shorten until they are 3 to 6 microns in length and 1 micron thick. During this shortening process a centromere constriction develops at some point along the length of each of the chromosomes (Fig. 2).

Chromosome morphology and number. The pre-metaphase chromosomes, prior to the appearance of any discernible lines of division, are relatively easy to analyze (Fig. 2). Later the analysis is more difficult because each chromosome continues to shorten as it divides (Fig. 5). Separation of the chromosomes is not uniform, and there is no characteristic attachment point at the centromeres, as seen in many mammalian preparations. The chromosomes lose the centromere constriction during anaphase as they continue to shorten. Figure 1 demonstrates a number of stained, unsectioned eggs in various stages of mitosis.

Repeated counts on over 200 pre-metaphase chromosome spreads of colchicine-treated eggs during the first cleavage division make it certain that the diploid chromosome number in *Arbacia punctulata* is 44. Moreover, in one experiment in which 5% of the controls showed dispermic cleavage, an examination of the chromosome spreads revealed about the same percentage of eggs that had 66 chromosomes. These triploid counts provide a further confirmation for the diploid count of 44.

The karyotype. Chromosomal length and centromere position provided the morphological criteria by which a karyotype (Fig. 6) was prepared from the chromosome spread of Figure 2. Table I provides a specification of the chromosome pairs into groups based on their morphological similarities.

Figure 3. Amphimixis in an unsectioned egg of *Arbacia punctulata*. The smaller, more condensed male pronucleus is coming into contact with the larger female pronucleus. The male pronucleus then flattens over the female pronucleus surface and gradual fusion occurs. Magnification, approximately 2000 ×.

Figure 4. A partially squashed prophase preparation of an *Arbacia* egg, demonstrating the coiling of the elongated chromatid threads. Oil immersion-phase contrast; magnification, approximately 2000 ×.

Figure 5. Metaphase chromosome spread of a colchicine-treated *Arbacia* egg in the first cleavage division. The chromosomes do not split in synchrony and there is no attachment at the centromere position. Oil immersion-phase contrast; magnification, approximately 2000 ×.

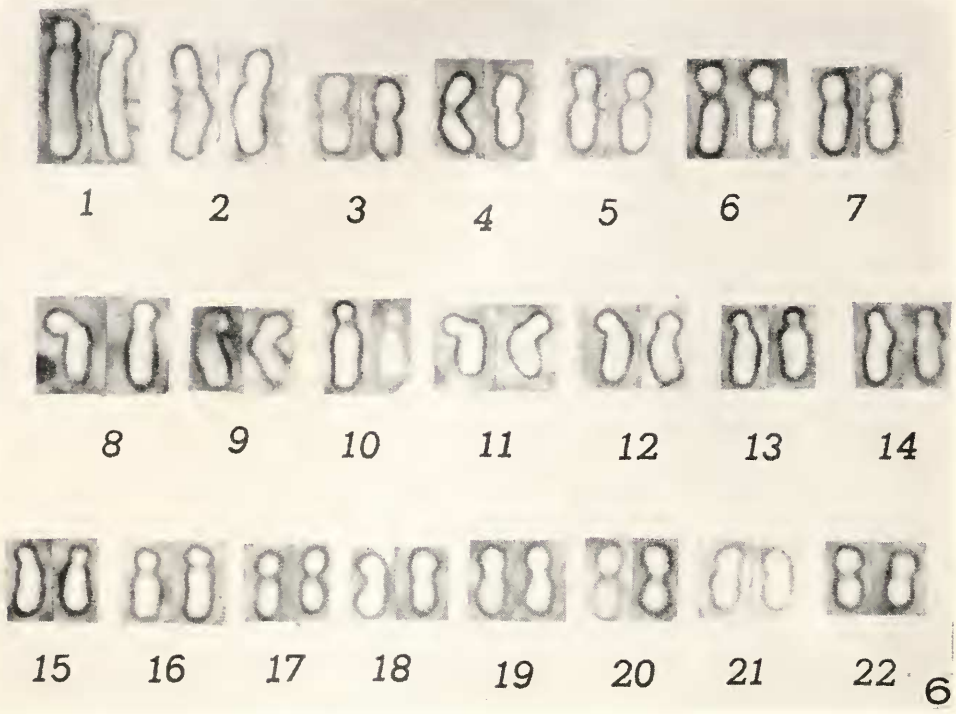


FIGURE 6. Karyotype obtained from the pre-metaphase chromosome spread preparation of Figure 2. Total magnification of the chromosomes, approximately 4000 ×.

Echinarachnius parma. The chromosomes of the egg of the sand dollar also were examined by this method. These much larger eggs are more difficult to study because of the wide area of dispersion of the chromosomes in the cytoplasm in spread preparations. Counts gave either 44 or 46 chromosomes for the diploid number. The chromosomes of the sand dollar are somewhat larger than those of the sea urchin, and the centromere position tends to be more medial. An unequivocal karyotype still cannot be determined with any accuracy.

TABLE 1

Karyotype of egg of Arbacia punctulata during the first cleavage division

Chromosome pair	Description
1	Large chromosomes, nearly terminal centromeres
2	Chromosomes not as large, more medial centromeres
3–4	Medium size chromosomes, approximately median centromeres
5–9	Medium size chromosomes, sub-median centromeres
10–14	Medium size chromosomes, nearly terminal centromeres
15–16	Smaller chromosomes, nearly terminal centromeres
17–19	Short chromosomes, sub-median centromeres
20	Short chromosomes, more medial centromeres
21–22	Very short chromosomes, approximately median centromeres

Discussion

A number of widely differing values have been estimated as the chromosome number of *Arbacia punctulata*. These vary from as low as 32 (Harvey, 1940) to as high as 40 (Tennent, 1912). In a discussion of these results, Harvey (1956, p. 102) concludes that most sea urchins, including *A. punctulata*, have 36 to 38 chromosomes, in agreement with the number reported by Matsui (1924). Baltzer (1910), however, found 40 chromosomes in a related species, *A. lixula*. It is important to realize that in all these early studies the chromosome counts were made on sectioned material, which introduces many difficulties and uncertainties.

More recent studies indicate a higher chromosome number, namely 42 to 44. Nishikawa (1961), on the basis of examining the meiotic divisions of spermatogonia, found a haploid number of 21, or a diploid value of 42, for two species of Japanese sea urchins, *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina*. The number, 44, reported in the present study, has also been confirmed recently by German (1964), who examined the chromosomes of *A. punctulata* in the blastula stage blastomeres by the standard acetic orcein squash technique. German described attenuation at the kinetochore region of the metaphase chromosomes, so that it is likely that the lack of centromere attachment in the present work is due to the new technique and is not characteristic of sea urchin chromosomes.

It may be that most echinoderms have a similar chromosome number and do not vary as widely as shown in the summary compiled by Makino (1951). One reason for this assumption is the chromosome number of *E. parma*, found in the present work to be 44 or 46, which is more in line with that found in *Arbacia*, and varies considerably from the earlier value of 52 determined by Matsui (1924).

The method here reported should prove useful to workers dealing with physical studies on marine eggs, especially *Arbacia*, during the early cleavage divisions. It makes it possible to determine, without sectioning the eggs, the degree of synchrony and the exact stage of mitosis (Fig. 1) at the time of an experimental treatment.

Summary

A method has been developed to stain the chromosomes and nuclei of unsectioned sea urchin and sand dollar eggs during the early cleavage divisions. The chromosome number of *Arbacia punctulata* is 44, and a karyotype of the pre-metaphase chromosomes of the first cleavage division has been made. The chromosome number of *Echinarachnius parma* is 44–46 and no karyotype has as yet been made.

Literature Cited

Auclair, W., 1964. On the chromosome number of *Arbacia*. *Biol. Bull.*, 127: 359.

Baltzer, F., 1910. Über die Beziehung zwischen dem Chromatin und der Entwicklung und Vererbungsrichtung bei Echinodermenbastarden. *Arch. f. Zellfor.*, 5: 497–621.

Fry, H. J., 1936. Studies of the mitotic figure. V. The time schedule of mitotic changes in developing *Arbacia* eggs. *Biol. Bull.*, 70: 89–99.

German, J., 1964. The chromosome complement of the blastomeres of *Arbacia punctulata*. *Biol. Bull.*, 127: 370–371.

Harris, P., 1961. Electron microscope study of mitosis in sea urchin blastomeres. *J. Biophy. Bioch. Cytol.*, 11: 419–431.

HARVEY, E. B., 1940. A comparison of the development of nucleate and non-nucleate eggs of *Arbacia punctulata*. *Biol. Bull.*, 79: 166–187.

HARVEY, E. B., 1956. The American Arbacia and Other Sea Urchins. Princeton University Press, Princeton, N. J.

MAKINO, S., 1951. An Atlas of the Chromosome Number in Animals. Iowa State College Press, Ames, Iowa.

MATSUI, K., 1924. Studies on the hybridization among the Echinoderms with special reference to the behavior of the chromosomes. *J. Coll. Agr. Imp. Univ. Tokyo*, 7: 211–236.

NISHIKAWA, S., 1961. Notes on the chromosomes of Echinoderms, *Hemicentrotus pulcherrimus* (A. Agassiz) and *Anthocidaris crassispina* (A. Agassiz). *Dobuts. Zasshi*, 70: 425–428.

TENNENT, D. H., 1912. Studies in cytology. I. A further study of the chromosomes of *Toxopneustes variegatus*. II. The behavior of the chromosomes in *Arbacia-Toxopneustes* crosses. *J. Exp. Zool.*, 12: 391–411.